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Amphiphysin, a neuronal protein first identified in chicken synaptic membranes, is the autoantigen of Stiff-Man Syndrome (SMS) associated with breast cancer. We have now cloned human amphiphysin and found the N- and C-terminal domains of the protein to be highly conserved between chicken and human. Patient autoantibodies have a distinct pattern of reactivity with amphiphysin, and the dominant autoepitope is located in its C-terminal region, which contains an SH3 domain. Portions of chicken and human amphiphysin are also homologous to portions of Rvs167 and Rvs161, two yeast proteins which are involved in cell entry into stationary phase upon exposure to unfavourable growth conditions.We have also demonstrated a specific, SH3 domain-mediated, interaction between amphiphysin and dynamin by gel overlay and affinity chromatography. In addition, we show that the two proteins are colocalized in nerve terminals and are coprecipitated from brain extracts consistent with their interactions *in situ*. We also report that a region of amphiphysin distinct from its SH3 domain mediates its binding to the α_C subunit of AP2 adaptin, which is also concentrated in nerve terminals. These findings support a role of amphiphysin in synaptic vesicle endocytosis.

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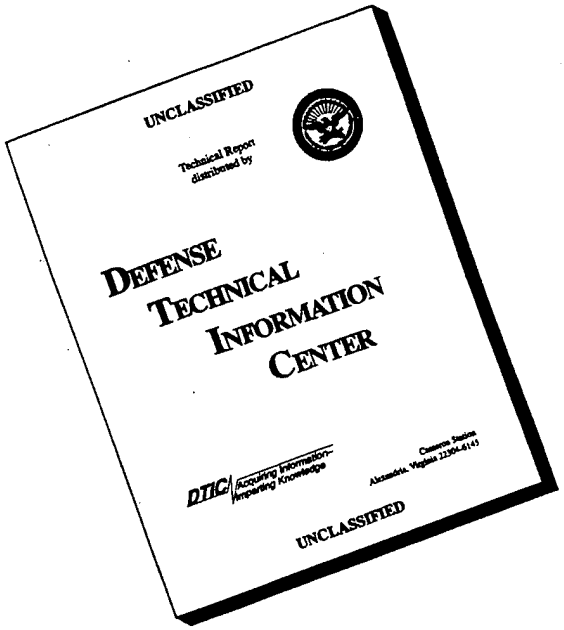

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Carol David 10/24/95

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INTRODUCTION

Stiff-man syndrome (SMS) is a rare neurological disease characterized by rigidity of the body musculature with superimposed painful spasms (Layzer, 1988). Most patients with this disease exhibit autoimmunity to GABA-ergic neurons. It has been found in Prof. De Camilli's laboratory that, to date, all patients diagnosed with breast cancer and SMS, have autoantibodies against a novel synaptic-associated protein, amphiphysin. This research project is to further define the role of anti-amphiphysin antibodies in the development and/or detection of breast cancer and to further understand the function of this protein and how the autoimmunity may arise.

In the early stages of the work on SMS, it was noticed in Prof. De Camilli's lab that two patients with this condition, but without GAD-antibodies or associated organ-specific autoimmune diseases, had high titers of autoantibodies directed against a 128 kDa protein. Immuno-cytochemistry suggested a synaptic localization of the autoantigen. Interestingly, both patients were women with breast cancer (ductal adenocarcinoma). Subsequently, the same antibodies were detected in a third patient with SMS without apparent breast cancer. On Prof. De Camilli's indication, a search of breast cancer in this patient was performed by ultrasonography and a small infiltrating ductal carcinoma was found and removed. Recently, a fourth case with the same triad (SMS, breast cancer and autoantibodies directed against the 128 kDa autoantigen) has been identified. Following publication of the report of the first three patients (Folli et al, 1993), it was learned of a fifth patient with SMS who died of breast cancer and who had been found positive for autoantibodies directed against a 128 kDa brain protein (D. Kaufman, Los Angeles, personal communication). In at least three of these cases (Folli et al, 1993; Meinck, personal communication) a remission of the neurological symptoms was documented after removal of the cancer and steroid therapy, supporting the hypothesis that no major degeneration of brain tissue occurs in SMS.

These findings raise the possibility that in some cases SMS may have an autoimmune paraneoplastic origin. Other autoimmune paraneoplastic neurological diseases have been described and characterized in recent years (Posner and Furneaux, 1990; Hetzel et al, 1990). These conditions are characterized by neurological symptoms which appear to follow the development of a cancer, and by the presence in the serum and CSF of high titer antibodies directed against specific brain autoantigen. The type of antibodies generally correlate with the type of neurological symptoms, but the pathogenetic role of these antibodies remains unclear. It was proposed that ectopic expression of brain antigens by cancer cells trigger the immune response (Furneaux et al, 1990).

Amphiphysin is a synaptic-vesicle-associated protein that was discovered by the screening of a λGT11 library of chicken brain with antibodies to synaptic proteins (Lichte et al., 1992). Its sequence (total of 682 amino-acids) includes a stretch of about 20 amino-acids which could potentially form a transmembrane span. However, most of the protein is cytosolic and only a pool of the protein interacts with the cytoplasmic surface of synaptic vesicles. Its function is unknown. The properties of amphiphysin suggested a possible identity with the 128 kDa antigen, a hypothesis that was tested and confirmed (De Camilli et al, 1993).

We have been able to clone human amphiphysin and found the N- and C-terminal domains of the protein to be highly conserved between chicken and human (David et al, 1994). Patient autoantibodies have a distinct pattern of reactivity with amphiphysin, and the dominant autoepitope is located in its C-terminal region, which contains an SH3 domain (David et al, 1994). Portions of chicken and human amphiphysin are also homologous to portions of Rvs167 and Rvs161, two yeast proteins which are involved in cell entry into stationary phase upon exposure to unfavourable growth conditions (David et al, 1994).

The work proposed here was geared to understand more about autoimmunity to amphiphysin and how it relates to breast cancer. The methods used concentrate on studying the function of amphiphysin using molecular biological and biochemical tools in addition to testing new patients with Stiff-Man Syndrome and breast cancer, for the presence of anti-amphiphysin antibodies.

BODY

Objective 1) To clone human amphiphysin; to prepare pure recombinant amphiphysin and poly- and monoclonal antibodies directed against amphiphysin

The work described below, on the cloning of human amphiphysin is summarized in David et al, 1994 (reprint attached).

In order to clone human amphiphysin, two fragments of chicken amphiphysin (Lichte et al, 1992), nt75-341 and nt777-1384, encoding for amino acids (a.a.) 1-81 and 228-430, respectively, were generated and tested for reactivity in Northern blot before proceeding to screen a λgt11 human cerebellar cDNA library. As expected, both fragments hybridized very strongly to a band of approximately 4.5 kb in chicken brain polyA$^+$ mRNA. They also labeled more weakly a band of similar size when tested under high stringency conditions on rat brain polyA+ mRNA.

The nt777-1384, corresponding to the central region of chicken amphiphysin (Lichte et al, 1992), was then used as a probe to screen 1.5 x 10^6 plaques of a λgt11 human cerebellar cDNA library. Eighteen positive clones were isolated, four of which (designated 22-2, 24, 27 and 34) were also positive when hybridized with the chicken fragment nt75-341, which encompassed the N-terminal. Sequence analysis revealed that clone 22-2 contained an ~2.4 kb insert, which had an open reading frame of 2088 nt (nt 111-2198) encoding a protein of 695 a.a.. The nucleotide sequence encoding the putative protein was 73% identical to the nucleotide sequence of chicken amphiphysin. The sequence surrounding the first ATG (gcagccatgg), at position 111 of clone 22-2, conformed very well to the initiation consensus sequence as defined by Kozak (1991). No polyadenylation signal was detected at the 3' end of the clone (nt 2199-2377). However, a stretch of 10 A's was found (nt 2365-2374), suggesting that internal annealing of the oligo(dT) primer occurred at this site. Clones 24, 27 and 34 were identical in sequence to smaller portions of clone 22-2 except for clone 27 which differed by 20 nucleotides in the 5' non-coding region. Whether this represents a cloning artifact or evidence of a transcript alternatively spliced at the 5' region, remains to be determined.

The predicted molecular weight (76.25 kDa) of the protein encoded by clone 22-2 was considerably smaller than the apparent molecular weight of rat amphiphysin in SDS-PAGE gels which is approximately 128 kDa (Folli et al, 1993; De Camilli et al, 1993). However, a similar aberrant electrophoretic mobility was previously reported for chicken amphiphysin (Lichte et al, 1992). Injection of a GST-human amphiphysin fusion protein in rabbits elicited the production of antibodies which reacted very strongly with the 128 kDa antigen recognized by patient sera in brain tissue. Additionally, the GST-fusion protein had an apparent molecular weight of approximately 160 kDa, of which only about 28 kDa could be attributed to GST. Finally, when the human and chicken sequences were aligned, the N- and C-termini of the two molecules were in precise register. In conclusion, the above data indicates that we had isolated a full-length clone of human amphiphysin.

Polyclonal antibodies directed against human amphiphysin were obtained by injecting rabbits with GST-human amphiphysin fusion protein (1 mg) which had been purified on a GTH-sepharose column followed by preparative SDS-PAGE. In addition, we have begun injecting mice with polyhistidine tagged amphiphysin for the production of monoclonal antibodies.

Objective 2: To characterize human anti-amphipysin antibodies

We received sera from a patient in Germany who had symptoms of Stiff-Man Syndrome and a suspect lump in her right breast. The sera was checked for the presence of anti-amphiphsin antibodies and was found to be positive for such antibodies. Surprisingly, during her biopsy, no neoplasm was found. She is now being closely monitored.

Objective 3) To study the function of amphiphysin

The work described below, on the function of amphiphysin is summarized in David et al, 1996 (preprint attached).

Strong evidence implicates the GTPase dynamin (Shpetner and Valle, 1989; De Camilli et al, 1995) in the internalization of synaptic vesicle membranes after exocytosis and, more generally, in internalization of clathrin-coated vesicles. Temperature-sensitive mutations of the dynamin gene (*shibire*) in *Drosophila* cause a selective arrest of the synaptic vesicle cycle at the stage of invaginated plasmalemmal pits (Kosaka and Ikeda, 1983; Koenig and Ikeda, 1989; Chen et al, 1991; Van der Bliek and Meyerowitz, 1991) and transfection of dominant negative dynamin mutants in fibroblastic cells block clathrin-mediated endocytosis (Van der Bliek et al, 1993; Herskovits et al, 1993). Recent studies have shown that dynamin forms rings at the neck of invaginated clathrin-coated vesicles and suggested that a conformational change of the rings which correlates with GTP hydrolysis leads to vesicle fission (Takei et al, 1995;Hinshaw and Schmid, 1995). The identification of dynamin's physiological binding partner will be an important next step towards a full elucidation of endocytotic mechanisms.

Dynamin has a proline-rich C-terminal region that binds to a subset of SH3 domains. It was found to bind most effectively to the SH3 domains of Grb2, phospholipase Cγ, and the p85 subunit of PI-3-kinase (Gout et al, 1993). However, none of these proteins was shown to be concentrated in nerve terminals and the significance of these interactions for synaptic vesicle recycling remains unclear. We have explored the possibility that amphiphysin, may represent a physiological partner for dynamin.

To this end, we performed a gel overlay assay with polyhistidine-tagged amphiphysin fusion protein. Amphiphysin bound selectively to a 100kDa protein. This protein had the same electrophoretic mobility as dynamin. A similar overlay experiment performed with GST fusion proteins of amphiphysin fragments (David et al, 1994) demonstrated that the domain of amphiphysin responsible for binding to the 100 kDa protein is contained within the C-terminal fragment of 150 amino acids, which includes the SH3 domain [GST-amph (SH3)].

In complementary experiments, this SH3-containing fragment of amphiphysin was used to affinity-purify binding proteins from a Triton X-100 extract of rat brain and the bound proteins were analyzed by Coomassie staining. Two major proteins were specifically absorbed by the column. The predominant protein, of 100 kDa, was confirmed by Western blotting to be dynamin. The other protein comigrated with the 145 kDa protein which, along with dynamin and synapsin I, is one of the three major brain Grb2-binding proteins in brain (McPherson et al, 1994a; 1994b). The SH3 domain of amphiphysin did not bind synapsin I (M_r ~ 80 kDa) as shown by both protein staining and Western blotting of the affinity purified material, demonstrating the unique specificity of amphiphysin's SH3 domain interactions. Taken together, these findings suggest that dynamin is a physiological binding partner for amphiphysin and that the two proteins may bind to each other *in situ.*

We therefore investigated whether dynamin could be co-immunoprecipitated with amphiphysin from rat brain extracts. Two rabbit antisera, CD5 and CD6, raised against a GST/amphiphysin fusion protein were used for these experiments. CD6, and to a much lesser extent CD5, co-immunoprecipitated dynamin from a Triton X-100 solubilized rat brain membrane extract as well as from rat brain cytosol. Another abundant brain protein, synapsin I (De Camilli et al, 1983), was not co-immunoprecipitated by either serum. The different immunoprecipitation properties of the two sera could be explained by the presence in CD5 of antibodies which compete with dynamin for binding to amphiphysin. Accordingly, while CD6 is directed against a central portion of the molecule (David et al, 1994), CD5 was found to react primarily with the C-terminal region of amphiphysin [GST-amph (SH3) fragment]. These findings are consistent with the possibility that dynamin and CD5 antibodies compete for the same binding sites on amphiphysin.

AP2 is a heterotetramer which participates in clathrin-mediated vesicle endocytosis from the plasmalemma (Robinson, 1994). Recently, it was reported that the appendage domain of the α subunit (α_c isoform) of AP2 binds amphiphysin and dynamin independently (19). Of the two α-adaptins, α_c is the only one which is expressed both in the brain and in other tissues while α_a is expressed exclusively in the brain (Ball et al, 1995). A possible physiological significance of the interaction between α_c-adaptin and amphiphysin is strengthened by the close colocalization of the two proteins in the nervous system, as assessed by double immunofluorescence.

In agreement with Wang et al (1995), α-adaptin present in brain extract, and preferentially α_c, was specifically retained on a column of full length amphiphysin This interaction was not indirect and mediated by dynamin, since the immobilized SH3 domain of amphiphysin (which does bind dynamin) did not retain α-adaptins. Both α_a- and α_c-adaptins have proline rich regions (Robinson, 1989) which could potentially bind directly to the SH3 domain of amphiphysin.

However, we did not detect α-adaptins in the brain material which was affinity purified by the GST-amph (SH3) fusion protein, by either protein staining or Western blotting with the monoclonal antibody AC1-M11 (22) that recognizes both α_a- and α_c-adaptins. In contrast, both α-adaptins were specifically retained by a GST-Grb2 fusion protein used as a control, but not by GST alone.

CONCLUSIONS

Since amphiphysin is a synaptic vesicle-associated protein (Lichte et al, 1992), its relation to a disease involving abnormal synaptic function is plausible. On the other hand, the connection between amphiphysin and breast cancer has been more elusive. A possible clue concerning this connection comes from an interesting homology we have identified by searching protein databases for a.a sequences similar to the regions of amphiphysin conserved between human and chicken. The conserved N-terminal region of amphiphysin has considerable homology to Rvs167 (Bauer et al, 1993) and Rvs161 (Crouzet et al, 1991), two yeast proteins which were cloned by isolating mutants with a reduced viability to nutrient starvation (rvs). While amphiphysin has been shown to have a very restricted tissue distribution (brain, endocrine tissues and testis) (Lichte et al, 1992; De Camilli et al, 1993), the presence of a homologue in yeast strongly suggests that amphiphysin homologues are present in all cells.

The phenotype of *RVS167* and *RVS161* mutant cells is associated with abnormal morphology and alterations in the peripheral cytoskeleton. Cells appear to be unable to adapt to unfavorable growth conditions by an impaired link between the mechanisms which control cell proliferation and those which allow the cell to undergo stationary phase adaptation. Mutations of either one of the two genes produce a similar phenotype (Crouzet et al, 1991; Bauer et al, 1993), and are suppressed by the same set of genes (Desfarges et al, 1993) indicating that they act in the same pathway. The function of amphiphysin in the nervous system remains to be elucidated. Amphiphysin was reported to be a synaptic vesicle-associated protein, although it is not enriched in these organelles (Lichte et al, 1992). The homology of amphiphysin to the two Rvs yeast proteins suggests a function of amphiphysin in controlling the properties of the membrane associated cytoskeleton and offers the possibility of using yeast genetics to further investigate the function of the protein.

It was proposed that neurological autoimmune paraneoplastic syndrome are triggered by the ectopic expression in the neoplastic tissues of a neuronal protein or a protein antigenically related to it, which then becomes an autoantigen (Thirkill et al, 1989; Furneaux et al, 1990). The homology of amphiphysin to yeast proteins which have been shown to participate in the cell adaptation to

stationary phase raises the possibility that amphiphysin or some related protein may be directly involved in at least some form of breast cancer. There is evidence to suggest that proteins of the peripheral cell cytoskeleton may be directly involved in the pathogenesis of some forms of cancer (Trofatter et al, 1993; Rubinfeld et al, 1993; Su et al, 1993). In the breast cancer tissue of SMS patients which have been investigated, amphiphysin immunoreactivity was not detected using patient autoantibodies (Folli et al, 1993). However, one should consider the possibility that the T-cell triggering autoepitope might belong to an amphiphysin-related molecule which similarly to Rvs161, contains only domain A. This protein would not be recognized by patient autoantibodies which we have now shown to be primarily directed against the C-terminus of amphiphysin. We note that Nova, another autoantigen of a paraneoplastic neurological autoimmune disorder, is a neuronal protein expressed in a truncated form (which does not include the dominant C-terminal autoepitope for humoral autoimmunity) in the neoplastic tissue (Buckanovich, et al, 1993).

In addition, the interaction of amphiphysin with dynamin and α-adaptin strongly links the function of amphiphysin to endocytosis. Independent support for a role of amphiphysin in endocytosis comes from genetic studies in yeast which express two amphiphysin homologues, Rvs167 and Rvs161 (Crouzet et al, 1991; Bauer et al, 1993). The two yeast proteins are hypothesized to form heterodimers, as mutations in the *RVS161* and *RVS167* genes produce a similar phenotype and are suppressed by the same set of genes (Desfarges et al, 1993; Munn et al, 1995). The mutant phenotype includes a striking endocytic defect (Munn et al, 1995) in addition to growth and polarity defects (Desfarges et al, 1993; Sivadon et al, 1995). The endocytic defect is characterized by impaired membrane internalization from the cell surface with a block of lucifer yellow uptake and a major impairment of α-factor receptor internalization (Munn et al, 1995). Yeast genetics will allow further testing of the hypothesis that some of the effects of the *RVS* genes on endocytosis are mediated by homologues of α-adaptins and dynamin. Additional effects of the *RVS* genes may be mediated by the interaction of the Rvs proteins with actin, as suggested by experiments carried out in the yeast two hybrid system (Amberg et al, 1995) and by genetic studies.

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Autoimmunity in Stiff-Man Syndrome with breast cancer is targeted to the C-terminal region of human amphiphysin, a protein similar to the yeast proteins, Rvs167 and Rvs161

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Abstract

Amphiphysin, a neuronal protein first identified in chicken synaptic membranes, is the autoantigen of Stiff-Man Syndrome (SMS) associated with breast cancer. We have now cloned human amphiphysin and found the N- and C-terminal domains of the protein to be highly conserved between chicken and human. Patient autoantibodies have a distinct pattern of reactivity with amphiphysin, and the dominant autoepitope is located in its C-terminal region, which contains an SH3 domain. Portions of chicken and human amphiphysin are also homologous to portions of Rvs167 and Rvs161, two yeast proteins which are involved in cell entry into stationary phase upon exposure to unfavourable growth conditions.

Key words: Stiff-Man Syndrome; Amphiphysin; Breast cancer; Synaptic vesicle; Rvs161; Rvs167

1. Introduction

Stiff-Man Syndrome (SMS) is a rare neurological disease characterized by rigidity of the body musculature with superimposed painful spasms [1–4]. SMS is one of the few human diseases for which evidence of an autoimmune process directed against CNS neuronal antigens has been found. High titer antibodies directed against neuronal autoantigens are found both in the serum and in the cerebrospinal fluid of the majority of SMS patients [5].

Two main targets of humoral autoimmunity have been identified in two populations of SMS patients which have similar neurological characteristics but different associated conditions. In 50–60% of the cases, autoantibodies are primarily directed against the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD). In these patients, insulin-dependent diabetes mellitus and other organ-specific autoimmune diseases are frequently present [5–7].

In five of the more than 100 cases of SMS patients whose sera we have tested, autoantibodies are directed against another neuronal protein of 128 kDa [8]. This protein was recently identified as amphiphysin [9], a synaptic vesicle-associated protein originally cloned from chicken brain [10]. Strikingly, all five patients with amphiphysin autoimmunity are women with breast cancer. In fact, in two of these patients, an infiltrating ductal adenocarcinoma of the breast was searched for, and found, only after the identification of the anti-amphiphysin antibodies ([8] and our most recent case (patient 1 of this study – see section 2)). A remission of the neurological symptoms was documented in three of the five patients after removal of the cancer and steroid therapy ([8,9,11] and this study (Dr. M. Myers, Jackson, MI, personal communication)) supporting the hypothesis that the condition results from a functional rather than structural damage of the CNS. Other cases of SMS associated with cancer have been described [12,13]. These findings raise the possibility that in some cases SMS may have an autoimmune paraneoplastic origin. As in the case of autoantigens of other autoimmune paraneoplastic diseases of the CNS, as well as of GAD, amphiphysin is an intracellular protein and the link between humoral autoimmunity directed against the autoantigen and the clinical symptoms remains to be explained.

In other neurological autoimmune paraneoplastic conditions, expression of the neuronal autoantigen in the tumor was reported [14,15]. It was proposed that the ectopic expression of the brain antigen by cancer cells may trigger the autoimmune response [16]. In some cases, the autoantigen is thought to play a role in neoplastic growth [17]. As a first step to investigate mechanisms of amphiphysin autoimmunity and a possible role of amphiphysin in the biology of human breast cancer, we have now cloned human amphiphysin and mapped the autoepitopes. By Western blotting, a stereotypic humoral autoimmune response to amphiphysin was detected similarly to what we have previously shown for

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Abbreviations: a.a., amino acid; cAmph, chicken amphiphysin; CNS, central nervous system; GAD, glutamic acid decarboxylase; hAmph, human amphiphysin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SMS, Stiff-Man Syndrome.

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GAD autoimmunity in SMS [18]. The region of amphiphysin most highly conserved between chicken and human is also similar to two yeast proteins, Rvs161 [19] and Rvs167 [20] which are implicated in the transition from exponential cell growth to stationary phase upon exposure to nutrient starvation. The similarity of amphiphysin to yeast proteins which participate in stationary phase adaptation suggests the possibility that amphiphysin, or a closely related protein, has a role in the biology of breast cancer.

2. Materials and methods

2.1. Human sera

A new serum, belonging to a 75-year-old woman (patient 1) with SMS was referred to us (Dr. S. Songcharden, Dr. M. Myers, Jackson, MI) to be tested for the presence of anti-neuronal antibodies. The detection of anti-amphiphysin autoantibodies prompted a search for an occult breast cancer. A small ductal adenocarcinoma was found and surgically removed. Sera from four patients with SMS and breast cancer were previously described (patients 2–5) [8,9]. Control sera were from healthy subjects.

2.2. DNA manipulations

Molecular biological procedures were performed according to standard protocols [21]. Synthetic oligonucleotides were synthesized (Keck Biotechnology Resource Laboratory, Yale University) in order to amplify via PCR from a λZAP chicken brain cDNA library (courtesy of M. Bartkiewicz and R. Baron, Yale University) two fragments of chicken amphiphysin [10] corresponding to nucleotides 75–341 and 777–1384 (nt75–341, nt777–1384). [α-^{32}P]dATP was incorporated into purified PCR products via primer-direct labeling as described by Bogue [22] and used as probes at 2×10^6 cpm/ml for Northern blots and 10^5 cpm/ml for library screening under relatively high stringency conditions (hybridization – 50% formamide, 6 × SSC, 0.1% SDS, 2 × Denhardt's, 100 mg/ml salmon sperm DNA, 37°C, 20 h; wash – 2 × SSC, 0.05% SDS, 45°C).

1.5×10^6 plaques of a λgt11 human cerebellar cDNA library (random- and oligo(dT)-primed; Clontech, Palo Alto, CA) were screened with nt777–1384. Phage cDNA inserts of positive clones were purified and sequenced [23]. Sequence data, hydrophilicity, and antigenicity profiles were compiled and analyzed using MacVector (IBI Ltd., Cambridge, UK) and MacDNASIS Pro (Hitachi, San Bruno, CA) software. BESTFIT [24], BLAST [25] and PILEUP [26] analyses were programs of the Genetics Computer Group (Madison, WI). Secondary structure predictions were done by the Self Optimized Prediction Method [27].

2.3. Preparation of fusion proteins

The fragment corresponding to nt 89–2377 of clone 22–2 was ligated into *Sma*I-*Eco*RI sites of pGEX-2T vector (Pharmacia, Piscataway, NJ) to generate a glutathione *S*-transferase (GST)/full-length human amphiphysin fusion protein. Clone 22–2 was used as a cDNA template in PCR reactions with Vent$_R$ DNA polymerase (New England Biolabs, Beverly, MA), to obtain the amphiphysin fragments encoding amino acids (a.a) 1–161, 132–291, 262–435, 411–581, and 545–695 (designated I–V, see Fig. 4A). *Sma*I and *Eco*RI sites were added to 5′ and 3′ ends of the fragments respectively to allow unidirectional subcloning into the polylinker region of pGEX-2T. The sequences of all GST-amphiphysin fusion constructs were confirmed by DNA sequencing. The fusion proteins were produced in DH5a cells and purified on a glutathione-Sepharose 4B column (Pharmacia, Piscataway, NJ) essentially as described [28]. Constructs yielded recombinant proteins composed of GST (~28 kDa) fused to the N-terminus of the corresponding amphiphysin fragment.

2.4. Miscellaneous procedures

SDS-PAGE of 5–16% gradient gels and Western blotting were performed essentially as described by Laemmli [29] and Towbin [30], respectively. Western blots with patient sera was performed as described [18].

A serum directed against human amphiphysin (CD6) was obtained by injecting a rabbit with GST-human amphiphysin fusion protein (1 mg) which had been purified on a GTH-Sepharose column followed by preparative SDS-PAGE.

3. Results and discussion

In order to clone human amphiphysin, two fragments of chicken amphiphysin [10], nt75–341 and nt777–1384, encoding for amino acids (a.a.) 1–81 and 228–430, respectively, were generated and tested for reactivity in Northern blot before proceeding to screen a λgt11 human cerebellar cDNA library. As expected, both fragments hybridized very strongly to a band of approximately 4.5 kb in chicken brain poly(A)$^+$ mRNA. They also labeled more weakly a band of similar size when tested under high stringency conditions on rat brain polyA+ mRNA (data not shown).

The nt777–1384, corresponding to the central region of chicken amphiphysin [10], was then used as a probe to screen 1.5×10^6 plaques of a λgt11 human cerebellar cDNA library. Eighteen positive clones were isolated, four of which (designated 22–2, 24, 27 and 34) were also positive when hybridized with the chicken fragment nt75–341, which encompassed the N-terminal. Sequence analysis revealed that clone 22–2 contained an ~2.4 kb insert, which had an open reading frame of 2088 nt (nt 111–2198) encoding a protein of 695 a.a. (Fig. 1, top line). The nucleotide sequence encoding the putative protein was 73% identical to the nucleotide sequence of chicken amphiphysin. The sequence surrounding the first ATG (gcagccatgg), at position 111 of clone 22–2, conformed very well to the initiation consensus sequence as defined by Kozak [31]. No polyadenylation signal was detected at the 3′ end of the clone (nt2199–2377). However, a stretch of 10 A's was found (nt2365–2374), suggesting that internal annealing of the oligo(dT) primer occurred at this site. Clones 24, 27 and 34 were identical in sequence to smaller portions of clone 22–2 except for clone 27 which differed by 20 nucleotides in the 5′ noncoding region. Whether this represents a cloning artifact or evidence of a transcript alternatively spliced at the 5′ region, remains to be determined.

The predicted molecular weight (76.25 kDa) of the protein encoded by clone 22–2 was considerably smaller than the apparent molecular weight of rat amphiphysin in SDS-PAGE gels which is approximately 128 kDa [8,9]. However, a similar aberrant electrophoretic mobility was previously reported for chicken amphiphysin [10]. Injection of a GST-human amphiphysin fusion protein in rabbits elicited the production of antibodies which reacted very strongly with the 128 kDa antigen recognized by patient sera in brain tissue (Fig. 2). Additionally, the GST-fusion protein had an apparent molecular

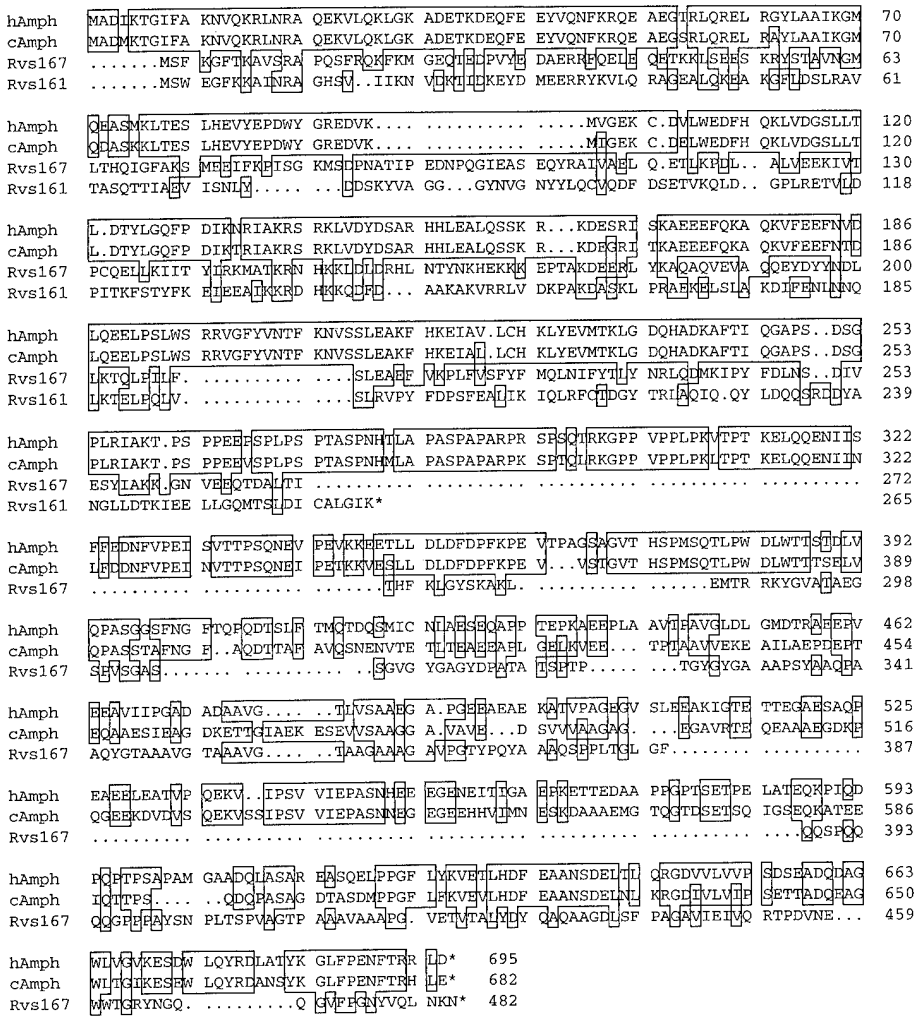


Fig. 1. Amino acid sequence alignment of human amphiphysin (hAmph) with chicken amphiphysin (cAmph) and the yeast proteins, Rvs167 and Rvs161. Amino acids are shown in single letter code and identity with the human sequence at a given position is boxed. First the sequences of the two amphiphysins and of Rvs167 were aligned to each other using the program PILEUP. The sequence of Rvs 161 was then compiled with the other three sequences according to its alignment with Rvs 167. The complete cDNA sequence of hAmph (clone 22–2) is available from GenBank under accession number U07616.

weight of approximately 160 kDa, of which only about 28 kDa could be attributed to GST (see figure 4B, panel CB, lane H). Finally, when the human and chicken sequences were aligned, the N- and C-termini of the two molecules were in precise register (Fig. 1, top two lines). In conclusion, the above data indicates that we had isolated a full-length clone of human amphiphysin.

A comparison of the a.a. sequences of human and chicken amphiphysin [10] revealed a high degree of similarity with the exception of a region of approximately 180 a.a. in the second half of the molecules (Fig. 1 and Fig. 3A domain C). Overall the two proteins are 75% identical and 84% similar at the a.a. level. As was previously reported for chicken amphiphysin [10], the human protein is very hydrophilic, has an acidic pI of 4.4, and contains many putative phosphorylation sites for protein kinases A and C and for casein kinase II. The first

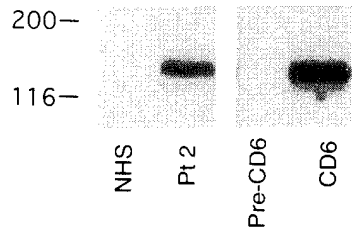
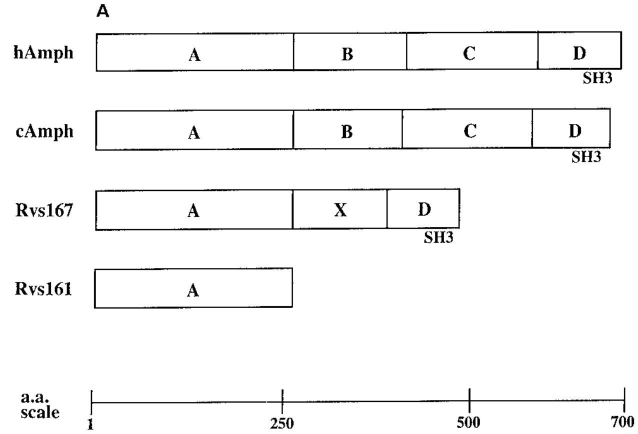


Fig. 2. The same protein band from rat brain is recognized by a rabbit antibody raised against human amphiphysin and by the serum of a patient with SMS and breast cancer. A total rat brain homogenate was subjected to SDS-PAGE and Western blotting as described in section 2. Sera used for western blotting are as follows: NHS = serum from control human subject, 1/500; Pt 2 = serum from patient with SMS and breast cancer, 1/500; pre-CD6 = pre-immune rabbit serum 1/50; CD6 = serum from rabbit immunized with human amphiphysin, 1/50. Molecular weight standards are indicated on the left.



B

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hAmph    TLHDFEAANS DELTLQRGDV VLVVPSDSEA DQDAGWLVGV KESDWLQYRD LATYKGLFPE NFTRRLD•     695
hsrc     A-Y-Y-SRTE TD-SFKK-ER LQI-...NNT EG-WWLAHSL STGQ...... ....T-YI-S -YV           140
yabp1    A-Y-YD--ED N---FVEN-K IINI...EFV -D-WWLGELE -DGS...... ....K----S -YVSLGN       592
hGRB2-C  A-F--DPQED G--GFR---F IH-M...DNS -P..N-WK-A CHGQ...... ....T*M--R -YVTPVNRNV•  217
Drk-C    A-Y--VPQE- G--DFR---- IT-T...DRS -E..N-WN-E IGNR...... ....--I--A TYVTPYHS      211
hspec    A-Y--Q-RSP R-VTMKK--- LTLL...-SI NK..D-WKVE AADH...... ....Q-IV-A -YV          1122
hGRB2-N  A-Y--K-TAD ---SFK---I LK-L...N-E CDQN.-YKAE LNGK...... ....D-FI*K -YIEMKPHDW    60
Drk-N    A-H--S-TAD ---SFRKTQI LKIL...NME -DSN.-YRAE LDGK...... ....E--I-S -YIEMKNHDW    60
Rvs167   A-Y-YQ-QAA GD-SFPA-A- IEI-...QRT PDVNE-WT-R YNGQ...... ....Q-V--G -YVQLNKN•    482

CONSENSUS ALYDF A  D  ELSF  GD  L    ...      DD   WW       G ...... .... G FP  NYV
           | || |     ||:   ||  :              |    |                   | ||  |:
hAmph    TLHDFEAANS DELTLQRGDV VLVVPSDSEA DQDAGWLVGV KESDWLQYRD LATYKGLFPE NFTRRLD•
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Fig. 3. (A) Domain diagram of amphiphysins and related yeast proteins suggested by blocks of a.a. similarity. The borders of the domains were based on sequence alignment and are drawn to scale (shown). (B) The last 60 a.a. of hAmph was aligned with the SH3 domains of human src (hsrc [42]), yeast abp1 (yabp1 [43]), human GRB2 (hGRB2 [35]), drosophila drk gene product (Drk [44]), human spectrin α chain (hspec [45]) and Rvs167 [20] using the PILEUP program. N and C represent N- and C-terminal SH3 domains where applicable. Numbers to the right of the sequence indicate the position of the last a.a. shown in the sequence of the protein and a (•) indicates a terminal a.a. Dashes indicate a.a. that are identical with human amphiphysin and periods represent gaps. A consensus SH3 sequence was constructed whenever the same a.a. was conserved in at least four of the sequences listed and an alignment of this sequence with amphiphysin is shown as was done by Bauer et al, [20]. G and P in bold refer to highly conserved residues and * indicates G_{203} and P_{49} of GRB2 (see text).

400 a.a. (domains A+B) are 91% identical and the last 100 a.a. (domain D) are 67% identical.

Although the C domains are only 40% identical, in this region the a.a. profile of chicken and human amphiphysin is strikingly similar with a predominance of alanine and glutamate. A proline-rich region between amino acids 263–277 in domain B, which contains putative binding sequences for src-homology (SH3) domains [32,33] is also highly conserved. SH3 domains are protien modules of about 60 a.a. that are found in many signaling and cytoskeletal proteins [34]. Domain C of chicken amphiphysin was reported to contain the only hydrophobic stretch (a.a. 478–499) of the molecule, although this stretch does not appear to form a transmembrane region [9,10]. A short a.a. sequence with similar hydrophobic properties is present in domain C of human amphiphysin (a.a. 468–480).

Domain D, present in both human and chicken amphiphysin, comprises the C-terminal 100 a.a. and is approximately 30% identical with SH3 domains from a variety of proteins in its last 60 a.a.(Fig. 3B). Secondary structure analysis of this domain predicted the presence of β-sheets between a.a. 648–652 and 664–668, in agreement with the known crystal structure of SH3 domains [34]. The gaps needed to align the SH3 domain consensus with amphiphysin are in regions where there is considerable variability between SH3 containing proteins [34]. Furthermore, the putative SH3 domain of amphiphysin includes a glycine (position 684) and a proline (position 687), (Fig. 3B – in bold) which are conserved in all SH3 domains listed. In particular, mutations of G_{203} or P_{49} of GRB2 (Fig. 3B, asterisks) result in an inability of GRB2 to bind other proteins via its C-terminal and N-terminal SH3 domains, respectively [28,35] and correspond to loss of function mutants in the GRB2 homologue, sem-5 [36].

The availability of the human amphiphysin clone allowed us to start investigating mechanisms of autoimmunity to amphiphysin in SMS associated with breast cancer. Neurological symptoms of SMS patients with either GAD or amphiphysin autoimmunity are similar [8,9]. This observation, together with the intracellular localization of both GAD and amphiphysin, suggest that autoantibodies are closely related to, but not responsible for, the disease. In the case of GAD autoimmunity, we have shown a common autoreactive pattern of SMS autoantibodies. In all cases, autoantibodies predominantly recognize a single epitope in the C-terminal region of GAD65 [18]. To determine whether the humoral autoimmune response to amphiphysin was stereotyped, sera from all 5 breast cancer patients with SMS as well as control sera were tested by western blotting against five overlapping amphiphysin fragments expressed as GST-fusion proteins (Fig. 4A).

As shown in Fig. 4B, autoantibodies from all 5 pa-

Table 1
Comparison of domain A of amphiphysin related proteins

	Chicken amphiphysin 1–261	Rvs 167 1–272	Rvs 161 1–265
Human amphiphysin 1–261	95.4 (97.7)	27.3 (48.7)	25.2 (49.2)
Chicken amphiphysin 1–261	–	26.5 (48.7)	22.8 (46.8)
Rvs 167 1–272	–	–	26.9 (51.9)

Percent identities and similarities (in brackets) of the a.a. sequence of domains A (numbers represent the boundaries of each domain A, refer to Fig. 3) of the indicated proteins as obtained using the BESTFIT program.

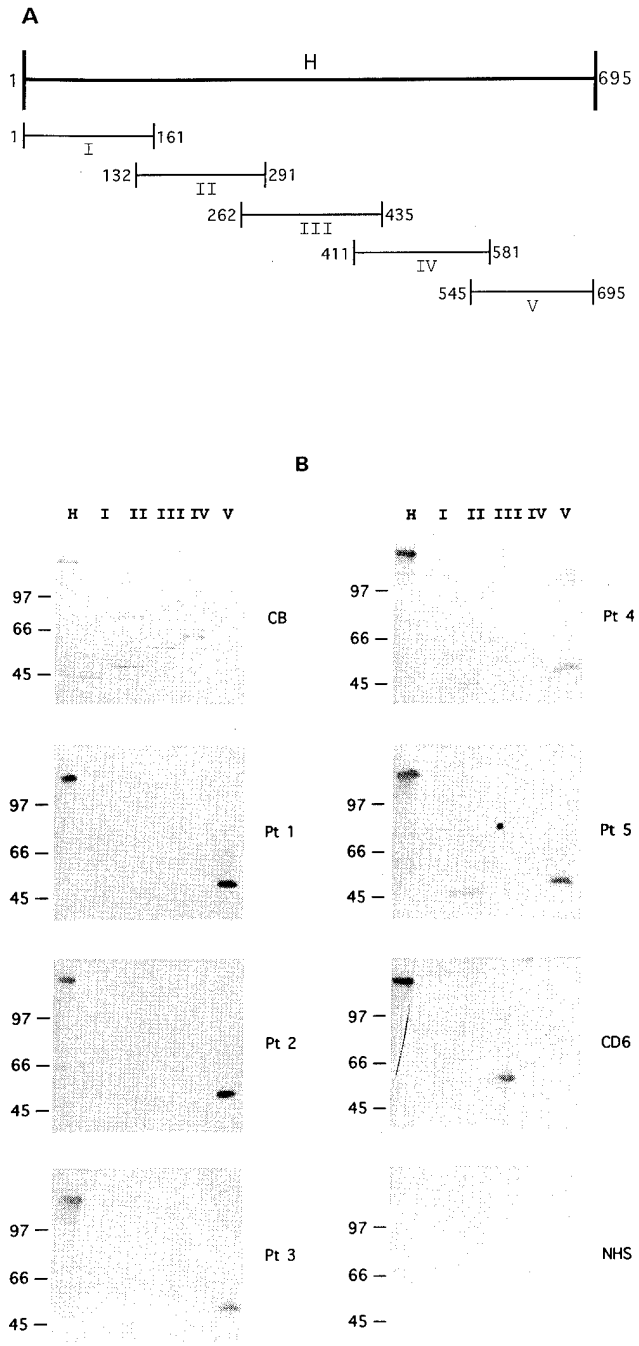

Fig. 4. Similar pattern of reactivity of patient sera with the C-terminal portion of human amphiphysin. (A) Schematic diagram of full-length human amphiphysin (H) and of its fragments (I–V) which were tested for reactivity with human sera. Boundaries of the fragments are indicated by a.a. numbers. (B) GST-fusion proteins of the constructs shown in A were subjected to SDS-PAGE and either Coomassie blue stained (CB) or Western blotted as described in section 2. The blots were reacted with either sera of patients with SMS and breast cancer (Pt 1–5), normal human sera (NHS), or CD6 (rabbit) sera at 1/500 as indicated. Approximately 1/3 of the amount loaded in the gel for panel CB was used for the Western blot analysis. These results are representative of at least 3 separate experiments. Molecular weight standards are indicated on the left. All fusion proteins (I–V) run between 45 and 66 kDa. The 80 kDa band seen in lane II (panel CB) was a protein from the host bacteria that was not related to the specific fusion protein, and never reacted with the antibodies.

tients were directed primarily, although not exclusively, against the amphiphysin C-terminal fragment (fragment V). Autoantibodies from patients 4 and 5 also recognized, but to a lower extent, fragment II. In contrast, a rabbit serum (CD6), raised against human amphiphysin, recognized primarily the central fragment III, suggesting that the stereotypic pattern of antibody reactivity against the C-terminal fragment is typical of the disease and does not simply reflect an unusually high antigenicity of this domain. We have previously reported that the neurological symptoms of SMS improve after removal of the cancer [8]. Interestingly, a similar improvement was noticed for the two patients identified after our original report (Dr. H.-M. Meinck, Heidelberg, Germany and Dr. M. Myers, Jackson, MI, personal communication). This observation strengthens the concept that SMS associated with breast cancer is an autoimmune paraneoplastic disease.

Since amphiphysin is a synaptic vesicle-associated protein [10], its relation to a disease involving abnormal synaptic function is plausible. On the other hand, the connection between amphiphysin and breast cancer has been more elusive. A possible clue concerning this connection comes from an interesting homology we have identified by searching protein databases for a.a sequences similar to the regions of amphiphysin conserved between human and chicken. The conserved N-terminal region of amphiphysin has considerable homology to Rvs167 [20] and Rvs161 [19], two yeast proteins which were cloned by isolating mutants with a *r*educed *v*iability to nutrient *s*tarvation (rvs). The alignment of Rvs167 and Rvs161 with human and chicken amphiphysin (Fig. 1) suggests a domain model of the four proteins shown in Fig. 3A. Domain A is shared by all four proteins and in all four has a high α-helix potential. The similarities among the various domains A are indicated in Table 1. Rvs161 is comprised exclusively of this domain, whereas Rvs167 shares an additional region of similarity with amphiphysin in domain D. Domain D of Rvs167 was previously shown to contain an SH3 domain [20]. Domain X of Rvs167 was defined as a GPA-rich region because of its high content in glycine, proline and alanine. While this region is substantially shorter than the central region of amphiphysin, it shares some features of both domains B and C of human and chicken amphiphysin which are rich in proline and alanine, respectively. These considerations suggest that Rvs167 is the yeast homologue of amphiphysin. While amphiphysin has been shown to have a very restricted tissue distribution (brain, endocrine tissues and testis)[9,10], the presence of a homologue in yeast strongly suggests that amphiphysin homologues are present in all cells.

The phenotype of *RVS167* and *RVS161* mutant cells is associated with abnormal morphology and alterations in the peripheral cytoskeleton. Cells appear to be unable to adapt to unfavorable growth conditions by an im-

paired link between the mechanisms which control cell proliferation and those which allow the cell to undergo stationary phase adaptation. Mutations of either one of the two genes produce a similar phenotype [19,20] and are suppressed by the same set of genes [37], indicating that they act in the same pathway. The function of amphiphysin in the nervous system remains to be elucidated. Amphiphysin was reported to be a synaptic vesicle-associated protein, although it is not enriched in these organelles [10]. The homology of amphiphysin to the two Rvs yeast proteins suggests a function of amphiphysin in controlling the properties of the membrane associated cytoskeleton and offers the possibility of using yeast genetics to further investigate the function of the protein.

It was proposed that neurological autoimmune paraneoplastic syndrome are triggered by the ectopic expression in the neoplastic tissues of a neuronal protein or a protein antigenically related to it, which then becomes an autoantigen [16,38]. The homology of amphiphysin to yeast proteins which have been shown to participate in the cell adaptation to stationary phase raises the possibility that amphiphysin or some related protein may be directly involved in at least some form of breast cancer. There is evidence to suggest that proteins of the peripheral cell cytoskeleton may be directly involved in the pathogenesis of some forms of cancer [39–41] In the breast cancer tissue of SMS patients which have been investigated, amphiphysin immunoreactivity was not detected using patient autoantibodies [8]. However, one should consider the possibility that the T-cell triggering autoepitope might belong to an amphiphysin-related molecule which similarly to Rvs161, contains only domain A. This protein would not be recognized by patient autoantibodies which we have now shown to be primarily directed against the C-terminus of amphiphysin. We note that Nova, another autoantigen of a paraneoplastic neurological autoimmune disorder, is a neuronal protein expressed in a truncated form (which does not include the dominant C-terminal autoepitope for humoral autoimmunity) in the neoplastic tissue [17].

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A ROLE OF AMPHIPHYSIN IN SYNAPTIC VESICLE ENDOCYTOSIS SUGGESTED BY ITS BINDING TO DYNAMIN IN NERVE TERMINALS

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(SH3 domain / adaptin / Grb2 / clathrin / RVS genes)

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Abstract

Amphiphysin, a major autoantigen in paraneoplastic Stiff-Man Syndrome, is an SH3 domain-containing neuronal protein, concentrated in nerve terminals. Here, we demonstrate a specific, SH3 domain-mediated, interaction between amphiphysin and dynamin by gel overlay and affinity chromatography. In addition, we show that the two proteins are colocalized in nerve terminals and are coprecipitated from brain extracts consistent with their interactions *in situ*. We also report that a region of amphiphysin distinct from its SH3 domain mediates its binding to the α_C subunit of AP2 adaptin, which is also concentrated in nerve terminals. These findings support a role of amphiphysin in synaptic vesicle endocytosis.

Introduction

Strong evidence implicates the GTPase dynamin (1,2) in the internalization of synaptic vesicle membranes after exocytosis and, more generally, in internalization of clathrin-coated vesicles. Temperature-sensitive mutations of the dynamin gene (*shibire*) in *Drosophila* cause a selective arrest of the synaptic vesicle cycle at the stage of invaginated plasmalemmal pits (3-6) and transfection of dominant negative dynamin mutants in fibroblastic cells block clathrin-mediated endocytosis (7,8). Recent studies have shown that dynamin forms rings at the neck of invaginated clathrin-coated vesicles and suggested that a conformational change of the rings which correlates with GTP hydrolysis leads to vesicle fission (9,10). The identification of dynamin's physiological binding partner will be an important next step towards a full elucidation of endocytotic mechanisms.

Dynamin has a proline-rich C-terminal region that binds to a subset of SH3 domains. It was found to bind most effectively to the SH3 domains of Grb2, phospholipase Cγ, and the p85 subunit of PI-3-kinase (11-14). However, none of these proteins was shown to be concentrated in nerve terminals and the significance of these interactions for synaptic vesicle recycling remains unclear. In this study we have explored the possibility that amphiphysin, a neuronal SH3-domain containing protein selectively concentrated in axon endings (15,16), may represent a physiological partner for dynamin. Amphiphysin is a hydrophilic, highly acidic protein, which is found both in soluble and particulate fractions of brain homogenates including synaptic vesicle membranes but is not enriched in purified synaptic vesicles (15,16). It is also an autoantigen in paraneoplastic, breast cancer-associated, Stiff-Man Syndrome (16-18). We show here that the SH3 domain of amphiphysin binds dynamin with high specificity and that the occurrence of this interaction *in situ* is supported by the close colocalization of the two proteins in neurons and by their coprecipitation from brain extracts.

In addition, we show that a recently reported interaction between amphiphysin and the α_C subunit of the plasmalemmal clathrin adaptor AP2 (19) is not mediated by amphiphysin's SH3 domain, although both α_A- and α_C-adaptins can link Grb2 via interactions which involve Grb2's SH3 domains.

These findings point to an important role of amphiphysin in synaptic vesicle endocytosis and suggest that it may participate in recruiting or regulating proteins involved in the budding and fission reactions.

Materials and Methods

Antibodies. Polyclonal antibodies (CD5 and CD6) directed against full-length GST-amphiphysin were raised in rabbits and affinity purified on His-amph fusion proteins. Polyclonal antibodies directed against dynamin were obtained by injecting rabbits with gel slices containing rat brain dynamin purified on a Grb2 column. A polyclonal anti-synapsin antibody (G246) was previously described (20). The T7 tag antibody which recognizes an 11 a.a. sequence in the pTrcHis constructs was from Novagen (Madison, WI). The following antibodies were generous gifts: anti-dynamin monoclonal antibodies Hudy-1 (21) from Dr. Sandra Schmid (Scripps Research Institute); anti α-adaptin monoclonal antibodies AC1-M11 (22) and AP.6 (23) from Dr. Margaret Robinson (University of Cambridge) and Dr. Francis Brodsky (UCSF) respectively.

Production and affinity purification of fusion proteins. Full-length polyhistidine-tagged amphiphysin (His-amph) was made by digesting GST-amphiphysin (18) with EcoRI and subcloning it into the pTrcHis C vector (Invitrogen, San Diego, CA). The resulting protein contained an N-terminal leader sequence that has six histidines in addition to an epitope recognized by the T7 tag antibody (Novagen, Madison, WI). His-rbSec1A [a.a. 2-593, subcloned into pTrcHis B (Invitrogen, San Diego, CA) at the EcoRI site] was a kind gift of Dr. Elizabeth Garcia from our laboratory (24). GST-amphiphysin (18) and GST-Grb2 fusion proteins (14) were produced and purified on a glutathione-sepharose 4B column (Pharmacia, Piscataway, NJ) as described (14). Polyhistidine-tagged fusion proteins were purified as recommended for the QIA express system using a Ni-NTA resin (QIAGEN, Chatsworth, CA).

Overlay assays. Nitrocellulose strips were blocked for one hour in 20mM Tris pH7.5, 150mM NaCl, 0.1% Tween-20 (TBST) + 5% powdered milk (Blotto) and then incubated with 4μg/ml of the indicated fusion protein in Blotto for 2h at room temperature. The nitrocellulose was then washed in TBST and bound proteins were detected with antibodies followed by ^{125}Protein A (NEN DuPont, Wilmington, DE).

Affinity-purification of solubilized brain extracts. For the preparation of total brain membrane extracts, frozen rat brains (Pel-Freeze Biologicals) were homogenized (1:10 w/vol.) using an SDT Tissumizer (Tekmar Co.) in 150 mM NaCl, 10 mM Hepes pH 7.4 containing pepstatin, aprotinin, leupeptin, antipain (each at 4 μg/ml), 0.4 mM PMSF, 10 mM benzamidine (HN buffer) and spun for 1 hr at 100,000xg. Pellets were solubilized in HN buffer containing 1% (vol/vol) Triton X-100 (Sigma)(HNT buffer), the mixture was incubated at 4°C for 1 hr with constant agitation, and insoluble material removed by centrifugation at 40,000xg for 1 hour at 4°C. Synaptosomal detergent extracts were prepared by solubilizing proteins from a rat brain P_2 fraction (24) in 50 mM $NaPO_4$, 20 mM HEPES, pH 7.4, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 0.83 mM

benzamidine, 0.23 mM PMSF at 4°C for 1 hr (14)(final protein concentration ~2 mg/ml). Detergent extracts were incubated with fusion proteins pre-bound to either glutathione-sepharose beads or Ni-NTA resin for 4hrs at 4°C. Beads were then washed with HNT buffer unless otherwise indicated and then eluted with SDS-PAGE sample buffer. Prior to binding to His-fusion proteins, the extracts were precleared by incubation with Ni-NTA beads for 1hr at 4°C.

Immunoprecipitation: Triton X-100 membrane extracts (see above) were precleared by incubation with Protein A sepharose CL-4B (Pharmacia, Piscataway, NJ) at 4°C for 1 hr. Antibodies were prebound to Protein A sepharose and then incubated with the precleared extracts for 4 hrs at 4°C. Beads were then washed three times with HNT buffer and bound proteins were eluted by boiling in sample buffer (14).

Miscellaneous procedures: Imunofluorescence of brain tissue was performed as described (20). SDS-PAGE of 5-16% gradient gels (unless otherwise indicated) and Western blotting were performed as described (14).

Results

An analysis of a variety of brain regions by double immunofluorescence demonstrated a striking colocalization of amphiphysin and dynamin as documented by Fig. 1. Panels *a* and *b* show two neurons in the brainstem, surrounded by immunoreactive nerve terminals. In addition, a diffuse immunoreactivity for both proteins is visible in the cytoplasm. Panels *c* and *d* demonstrate the colocalization of amphiphysin and dynamin in the cerebellar cortex where both proteins are present at particularly high concentrations in the basket cell nerve terminals (arrows) which surround the axon hillocks of Purkinje cells. The similar localization of dynamin and amphiphysin is consistent with their interaction.

To explore the possibility of a direct binding of amphiphysin to dynamin, we performed a gel overlay assay with polyhistidine-tagged amphiphysin fusion protein. As shown in Fig. 2, amphiphysin (a), but not a control polyhistidine-tagged protein (RbSec1)(c), bound selectively to a 100kDa protein. This protein had the same electrophoretic mobility as dynamin (b). A similar overlay experiment performed with GST fusion proteins of amphiphysin fragments (18) demonstrated that the domain of amphiphysin responsible for binding to the 100 kDa protein is contained within the C-terminal fragment of 150 amino acids, which includes the SH3 domain [GST-amph (SH3)] (not shown).

In complementary experiments, this SH3-containing fragment of amphiphysin was used to affinity-purify binding proteins from a Triton X-100 extract of rat brain and the bound proteins were analyzed by Coomassie staining (Fig. 3A). Two major proteins were specifically absorbed by the column. The predominant protein, of 100 kDa, was confirmed by Western blotting to be dynamin (Fig. 3B). The other protein comigrated with the 145 kDa protein which, along with dynamin and synapsin I (20), is one of the three major brain Grb2-binding proteins in brain (14,25). The SH3 domain of amphiphysin did not bind synapsin I (M_r ~ 80 kDa) as shown by both protein staining

(Fig. 3A) and Western blotting of the affinity purified material (Fig. 3B), demonstrating the unique specificity of amphiphysin's SH3 domain interactions. Taken together, these findings suggest that dynamin is a physiological binding partner for amphiphysin and that the two proteins may bind to each other *in situ.*

We therefore investigated whether dynamin could be co-immunoprecipitated with amphiphysin from rat brain extracts. Two rabbit antisera, CD5 and CD6, raised against a GST/amphiphysin fusion protein were used for these experiments. CD6, and to a much lesser extent CD5, co-immunoprecipitated dynamin from a Triton X-100 solubilized rat brain membrane extract (Fig. 4) as well as from rat brain cytosol (not shown). Another abundant brain protein, synapsin I (20), was not co-immunoprecipitated by either serum (Fig. 4, right panel). The different immunoprecipitation properties of the two sera could be explained by the presence in CD5 of antibodies which compete with dynamin for binding to amphiphysin. Accordingly, while CD6 is directed against a central portion of the molecule (18), CD5 was found to react primarily with the C-terminal region of amphiphysin [GST-amph (SH3) fragment](not shown). These findings are consistent with the possibility that dynamin and CD5 antibodies compete for the same binding sites on amphiphysin.

AP2 is a heterotetramer which participates in clathrin-mediated vesicle endocytosis from the plasmalemma (26). Recently, it was reported that the appendage domain of the α subunit (α_C isoform) of AP2 binds amphiphysin and dynamin independently (19). Of the two α-adaptins, α_C is the only one which is expressed both in the brain and in other tissues while α_A is expressed exclusively in the brain (27). A possible physiological significance of the interaction between α_C-adaptin and amphiphysin is strengthened by the close colocalization of the two proteins in the nervous system, as assessed by double immunofluorescence (Fig. 5).

In agreement with Wang et al (19), α-adaptin present in brain extract, and preferentially α_C, was specifically retained on a column of full length amphiphysin (Fig. 6, lane d). This interaction was not indirect and mediated by dynamin, since the immobilized SH3 domain of amphiphysin (which does bind dynamin [Fig. 3]) did not retain α-adaptins (Fig. 6). Both α_A- and α_C-adaptins have proline rich regions (28) which could potentially bind directly to the SH3 domain of amphiphysin. However, we did not detect α-adaptins in the brain material which was affinity purified by the GST-amph (SH3) fusion protein, by either protein staining (Fig. 3A) or Western blotting with the monoclonal antibody AC1-M11 (22) that recognizes both α_A- and α_C-adaptins (Fig. 6, lane b). In contrast, both α-adaptins were specifically retained by a GST-Grb2 fusion protein used as a control (Fig. 6, lane a), but not by GST alone (Fig. 6, lane c).

Although Grb2 has been found to bind dynamin as well as a variety of membrane proteins which are concentrated at clathrin-coated pits (29,30), the interaction between the subunits

of AP2 and Grb2 (Fig. 6) has not been reported previously. We further characterized the Grb2-AP2 interaction. Fig. 7 shows that this interaction involves the SH3 domains of Grb2 because a mutant Grb2 which has no SH3 domain function (29,14), cannot bind either α_a- or α_c-adaptin. Since α-adaptins are not retained on the immobilized SH3 domain of amphiphysin (which does bind dynamin), the interaction between Grb2 and AP2, which may be indirect (see also Fig. 8), is not mediated by dynamin.

Discussion

In this paper we demonstrate that amphiphysin is an important binding partner for dynamin, the key protein involved in the fission of synaptic vesicles (5,6,9) and more generally of clathrin-coated vesicles (7,8,21) from the plasmalemma.

A direct and specific interaction between dynamin and the SH3 domain of amphiphysin was demonstrated by *in vitro* studies. The binding properties of the SH3 domain of amphiphysin appear to be unique as other SH3 domain containing proteins have clearly different binding characteristics. Sequence analysis of the SH3 domain of amphiphysin includes a predicted insert (a.a. 673-683) which is unique when compared to other SH3 domains (18) and may help to define its specificity. Two criteria which are critical to validate the physiological significance of an interaction are fulfilled for the dynamin-amphiphysin interaction: (1) the two proteins are localized in the same cellular compartment and (2) significant amounts of dynamin can be coprecipitated with amphiphysin. Although dynamin binds *in vitro* to several SH3 domain containing proteins (11,31), amphiphysin is the only SH3 containing protein for which both these criteria are fulfilled. Thus, we speculate that amphiphysin is a major dynamin binding partner *in vivo*.

We also corroborate the recent finding that amphiphysin interacts with α_c subunit of AP2 adaptin (19), a protein of the plasmalemma clathrin coat (26), and we demonstrate that this binding is mediated by a site on amphiphysin distinct from the dynamin binding site. Finally, we demonstrate a novel, SH3-mediated, possibly indirect, interaction between AP2 and Grb2, an SH3/SH2 domain containing protein implicated in mediating downstream effects of tyrosine-phosphorylated receptors (29,30,32). Since these receptors are internalized by clathrin-coated pits, this result has the potential to be of physiological relevance.

The interaction of amphiphysin with dynamin and α-adaptin strongly links the function of amphiphysin to endocytosis. A pool of both amphiphysin and dynamin is present in a clathrin-coated vesicle preparation. However, in contrast to α-adaptins, neither protein co-enriches with clathrin and AP2 subunits in this fraction ([9] and unpublished observations). These findings suggest that amphiphysin, like dynamin, is not a major component of the clathrin coat and that it may have an accessory function in clathrin-coated vesicle endocytosis.

Independent support for a role of amphiphysin in endocytosis comes from genetic studies in yeast which express two amphiphysin homologues, Rvs167 and Rvs161 (18,33,34). The two yeast proteins are hypothesized to form heterodimers, as mutations in the *RVS161* and *RVS167* genes produce a similar phenotype and are suppressed by the same set of genes (35,36). The mutant phenotype includes a striking endocytic defect (36) in addition to growth and polarity defects (34,35,37). The endocytic defect is characterized by impaired membrane internalization from the cell surface with a block of lucifer yellow uptake and a major impairment of α-factor receptor internalization (36). Yeast genetics will allow further testing of the hypothesis that some of the effects of the *RVS* genes on endocytosis are mediated by homologues of α-adaptins and dynamin. Additional effects of the *RVS* genes may be mediated by the interaction of the Rvs proteins with actin, as suggested by experiments carried out in the yeast two hybrid system (38) and by genetic studies (36). Generally, studies in yeast have suggested an important role of the actin-based cytoskeleton in endocytosis (39).

These observations support the following model of synaptic vesicle endocytosis (Fig. 8). First the AP2 complex is recruited at the cell surface via interactions with components of the plasmalemma. These interactions may include the binding of AP2 to the cytoplasmic tail of synaptotagmin (40,41) and to other plasmalemmal components (42) which may serve to produce sufficient concentration of AP2 to facilitate formation of an AP2 lattice. Bound AP2, in turn, acts as a template for clathrin oligomerization and mediates a concentration of dynamin in proximity of the clathrin coats either via a direct interaction (19) or via amphiphysin and possibly Grb2 which are both dynamin-binding proteins (11,12,14). Our results show that amphiphysin could feasibly bind dynamin and α-adaptin simultaneously, since the binding occurs at two different regions of amphiphysin. Likewise, Grb2, which has two SH3 domains, could link dynamin to AP2, although the rather low concentration of Grb2 in brain (our unpublished observations) suggests that it does not play a major role in synaptic vesicle endocytosis. Neither dynamin nor amphiphysin are intrinsic components of clathrin coats, but a concentration of dynamin at clathrin coats was demonstrated by immunogold cytochemistry (9,21). A local high concentration of dynamin at clathrin coats, mediated by the multiple interactions described above, may facilitate oligomerization of a dynamin ring as soon as rearrangement of the clathrin coat has generated a narrow stalk. Clearly, this model must be further tested by studies on intact cells and in cell free systems. However, our data, together with the homology of amphiphysin to the RVS genes, strongly link the function of amphiphysin to endocytosis.

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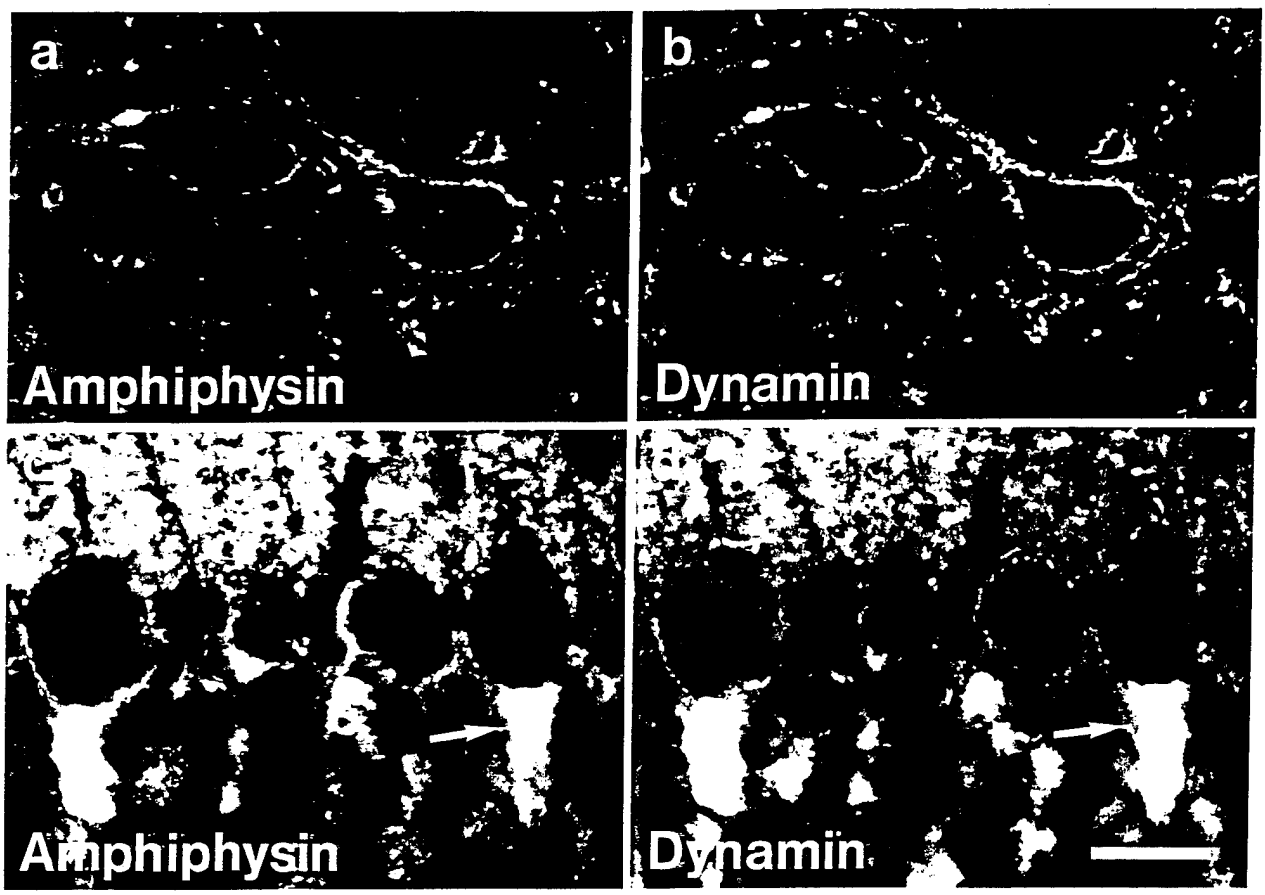

Figure 1: Colocalization of amphiphysin and dynamin in rat brain. Frozen sections of the brainstem (*a* and *b*) and of the cerebellum (*c* and *d*) stained by double immunofluorescence for amphiphysin (antibody CD5) and for dynamin (monoclonal antibody Hudy-1). Immunoreactive puncta, representing individual nerve terminals, outline two large perikarya in fields *a* and *b*. Large accumulation of immunoreactivity at the bottom of Purkinje cells (arrows) in fields *c* and *d* represent terminals of basket cell axons. Bar = 22.2 μm (*a* and *b*) and 26.0 μm (*c* and *d*).

Figure 1

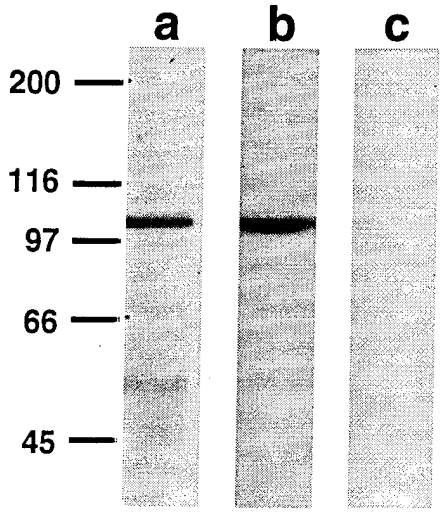

Figure 2: Amphiphysin binds selectively in a gel overlay assay to a 100 kDa protein which comigrates with dynamin. Rat brain homogenates were separated by SDS-PAGE, transferred to nitrocellulose, overlaid as indicated with either full-length His-amphiphysin (*a*) or His-rbSec1A (*c*), and subsequently with the T7 tag antibody to reveal His-tagged protein. A third gel lane (*b*) was reacted with a polyclonal antibody directed against dynamin. Molecular weight standards are indicated on the left.

Figure 2

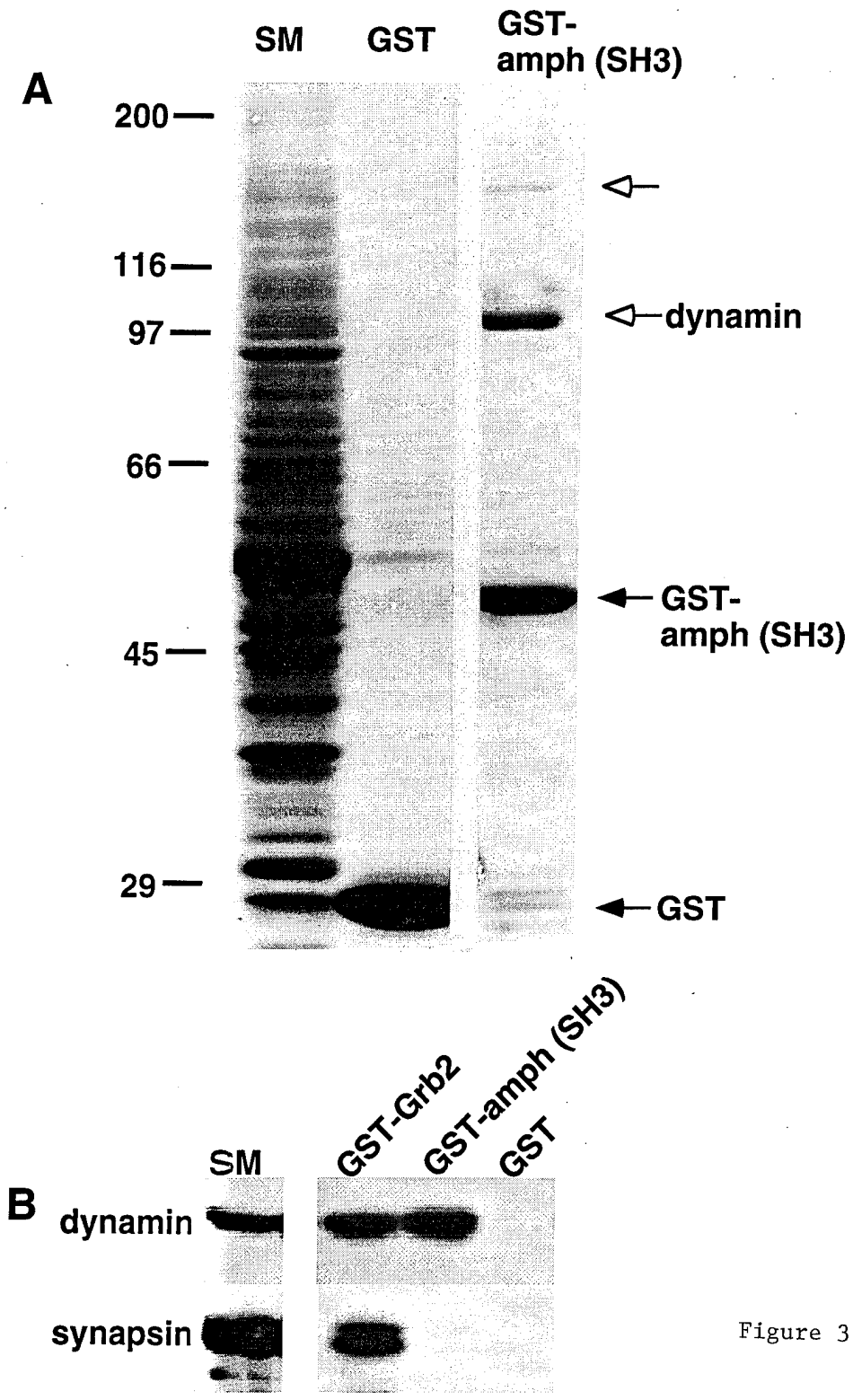

Figure 3

Figure 3: Affinity-purification of a rat brain extract on the SH3 domain of amphiphysin. A: Triton X-100 solubilized membrane proteins (~750 µg) were incubated with 20 µl glutathione beads preadsorbed either with (~40 µg) GST alone or with a GST fusion protein comprising the C-terminal fragment of amphiphysin [GST-amph (SH3)]. The material bound to the beads was eluted with SDS-PAGE sample buffer, run on SDS-PAGE in parallel with the starting material (SM) and stained with Coomassie brilliant blue. Two major bands of 100 kDa (dynamin) and 145 kDa (open arrow) were affinity-purified specifically. B: The fractions shown in A and the material affinity-purified by GST-Grb2 beads using identical affinity-purification conditions were reacted by western blotting for dynamin and synapsin I.

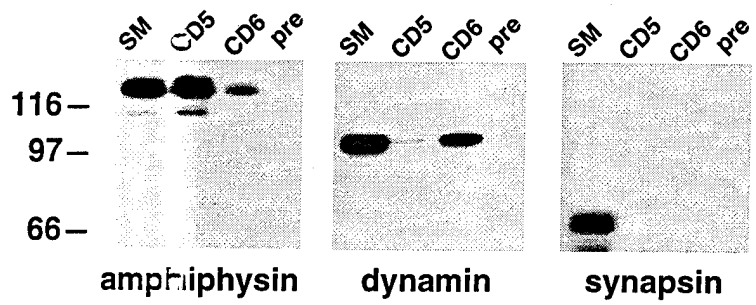

Figure 4: Co-immunoprecipitation of dynamin with amphiphysin from rat brain extracts. Amphiphysin was immunoprecipitated from Triton X-100 solubilized rat brain membranes with either CD5 or CD6 serum, or with a pre-immune serum (pre) as indicated. Starting material (SM) and immunoprecipitates were analyzed by western blotting for the proteins indicated.

Figure 4

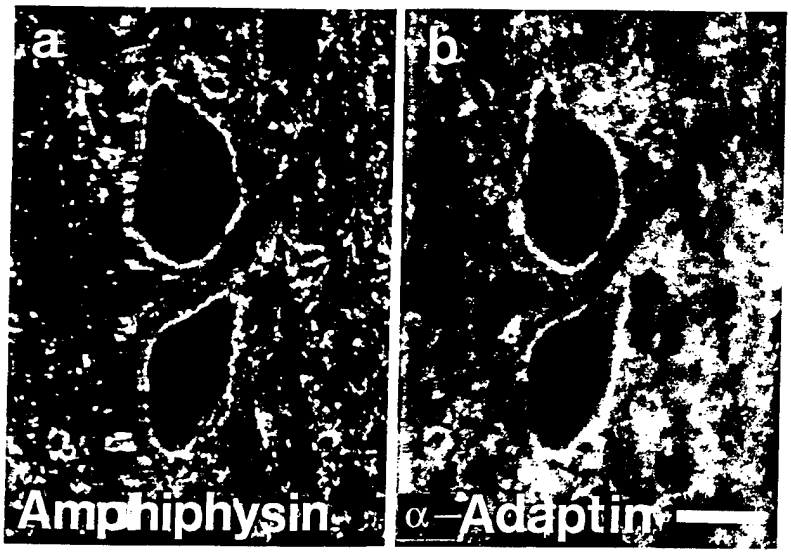

Figure 5: Colocalization of amphiphysin and α-adaptin in brain. *a* and *b*: Rat brain stem section stained by double immunofluorescence for amphiphysin (antibody CD5) and α-adaptin (monoclonal antibody AP.6). Bar = 19.5 μm.

Figure 5

Figure 6: α_c-adaptin binds to amphiphysin at a regions distinct from its SH3 domain. Triton X-100 solubilized rat brain membrane proteins (~750 µg) were incubated with 20 µl glutathione-Sepharose (for GST constructs) or Ni-sepharose (for His constructs) preadsorbed with the ~40 µg of GST-Grb2 (*a*), GST-amph (SH3) (*b*), GST (*c*), or HIS-amph (*d*). The material bound to the beads was analyzed by western blotting for α-adaptin (monoclonal antibody AC1-M11). α_a and α_c represent α-adaptin isoforms .

Figure 6

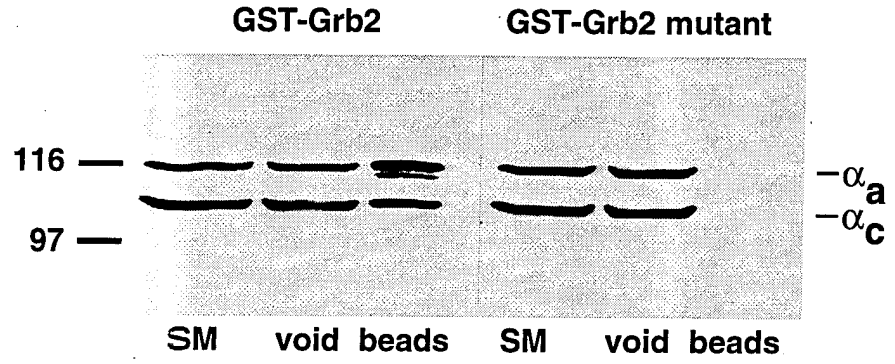

Figure 7: α-adaptins bind to the SH3 domain of Grb2. Triton X-100 solubilized proteins from a rat brain synaptosomal fraction (~750 μg) were incubated with 25 μl of glutathione-Sepharose preadsorbed with (15 μg) Grb2/GST fusion protein (GST-Grb2) or mutant Grb2/GST fusion protein (GST-Grb2 mutant) harboring a point mutation in each of the two SH3 domains. Equal aliquots of the material bound to the beads was eluted with SDS-PAGE sample buffer and reacted by western blotting for α-adaptins in parallel with the starting material (monoclonal antibody AC1M11).

Figure 7

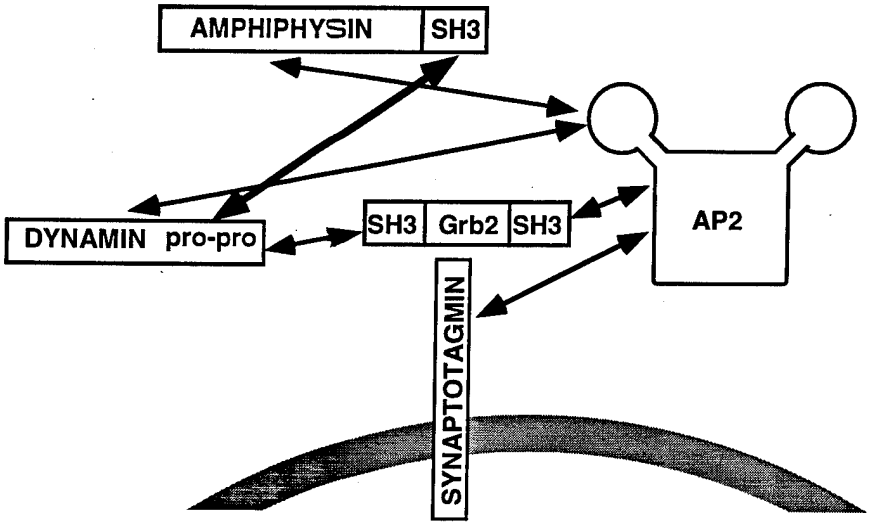

Figure 8: Model of dynamin-amphiphysin interaction (bold arrow) and additional interactions discussed in the text. The interactions illustrated may help to create a local concentration of dynamin in proximity of plasmalemmal clathrin coats which facilitate dynamin oligomerization into rings at the neck of the invaginated coated vesicles.

Figure 8